PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Tatsuo KAKIMOTO, et al.

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For: ANALYSIS OF AGONIST-ACTIVITY AND ANTAGONIST-ACTIVITY TO CYTOKININ RECEPTOR

DECLARATION UNDER 37 C.F.R. § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, TSUTOMU INOUE, do hereby declare and state:

THAT Tatsuo Kakimoto, Masayuki Higuchi, and I are the inventors of the subject matter disclosed and claimed in the above-mentioned application;

THAT we are co-authors of *Nature* Vol. 409, 1060-1063 (2001) (a copy of which is attached); and

THAT the present invention was invented prior to October 16, 2000, as evidenced by the date that the manuscript published as *Nature* Vol. 409, 1060-1063 was received by the Journal Nature for publication (see page 1063, above references). *Nature* Vol. 409, 1060-1063 shows typical working examples of the present invention (see page 1061, right column, lines 15-36, and page 1062, Figure 4). *Nature* Vol. 409, 1060-1063 shows *CRE1* gene, which is a typical example of a cytokinin receptor gene within scope of the claims. *Nature* Vol. 409, 1060-1063 also shows a yeast strain deficient in the *SLN1* gene (*sln1 Δ* mutant) (page 1061, right column, lines 15-26), which is a typical example of "a host cell having a lowered intrinsic histidine kinase activity, wherein said intrinsic histidine kinase activity was lowered by the defect in one or more histidine

kinase genes". Furthermore, *Nature* Vol. 409, 1060-1063 shows a *sln1 Δ* mutant carrying p415CYC-CRE1 (page 1061, right column, lines 26-27), which is a typical example of "a cell transformed with DNA comprising a cytokinin receptor gene, wherein the transformed cell expresses said cytokinin receptor from said DNA, and wherein growth of said transformed cell is controlled by intracellular signal transduction from said cytokinin receptor". Moreover, *Nature* Vol. 409, 1060-1063 shows a method for determining a level of intracellular signal transduction by measuring growth of said transformed cell in presence of examinee substance (page 1061, right column, lines 28-29), and determining a second level of intracellular signal transduction by measuring growth of said transformed cell in absence of said examinee substance (page 1061, right column, lines 26-27). *Nature* Vol. 409, 1060-1063 further shows comparing said level and said second level of intracellular signal transduction from said cytokinin receptor (page 1061, right column, lines 26-36, and page 1062, Figure 4), Thus, *Nature* Vol. 409, 1060-1063 shows typical working examples of the claimed method for determining agonist-activity to a cytokinine receptor.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 or the United States Code, and that such willful false statements by jeopardize the validity of this application or any patent issuing thereon.

Date: _____ Name: _____
TSUTOMU INOUE

Identification of CRE1 as a cytokinin receptor from *Arabidopsis*

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Cytokinins are a class of plant hormones that are central to the regulation of cell division and differentiation in plants[1,2]. It has been proposed that they are detected by a two-component system, because overexpression of the histidine kinase gene *CKI1* induces typical cytokinin responses[3] and genes for a set of response regulators of two-component systems can be induced by cytokinins[4,5]. Two-component systems use a histidine kinase as an environmental sensor and rely on a phosphorelay for signal transduction. They are common in microorganisms, and are also emerging as important signal detection routes in plants[6–9]. Here we report the identification of a cytokinin receptor. We identified *Arabidopsis cre1* (*cytokinin response 1*) mutants, which exhibited reduced responses to cytokinins. The mutated gene *CRE1* encodes a histidine kinase. *CRE1* expression conferred a cytokinin-dependent growth phenotype on a yeast mutant that lacked the endogenous histidine kinase SLN1 (ref. 10), providing direct evidence that CRE1 is a cytokinin receptor. We also provide evidence that cytokinins can activate CRE1 to initiate phosphorelay signalling.

Generally, cytokinins induce cell division, chloroplast development and formation of shoots (buds)[1]. We screened mutagenized *Arabidopsis* for mutants that were impaired in cytokinin responses, including rapid cell proliferation and shoot formation in tissue culture. We isolated a mutant designated *cytokinin response 1-1* (*cre1-1*). We tested the responses of *cre1-1* to auxin and cytokinin in tissue culture, using naphthalene acetic acid (NAA) as an auxin and kinetin as a cytokinin (Fig. 1). Wild-type explants responded to increasing levels of kinetin with rapid proliferation, greening and formation of shoots (Fig. 1a). By contrast, such cytokinin responses were not evident in *cre1-1* (Fig. 1b). The mutant was also less responsive to other cytokinins, including *trans*-zeatin, isopentenyladenine, benzyl adenine and the phenylurea-type synthetic cytokinin thidiazuron (see Supplementary Information).

Next we tested the responses of *cre1-1* to various plant hormones in a root elongation assay. External application of cytokinins[11], ethylene[12], auxins[13] or abscisic acid[14] inhibits root elongation. The root of the *cre1-1* mutant was less sensitive to benzyl adenine than that of wild-type plants, but it responded normally to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and the auxin indole-3-acetic acid (IAA) (Fig. 2a–c). The responses of *cre1-1* to low levels of abscisic acid (ABA) were slightly higher than normal (Fig. 2d). The cytokinin responses of *cre1-1* heterozygotes were intermediate between those of *cre1-1* homozygotes and the wild type (see Supplementary Information).

We mapped the *CRE1* locus to the top of chromosome 2 between the rga and nga1145 markers (see Supplementary Information). We searched the genome sequence of *Arabidopsis* between these markers for genes that could code for proteins involved in signal transduction. Among them was the hypothetical gene *At2g01830*, possibly coding for a histidine kinase. The nucleotide sequence of *At2g01830* revealed that this gene was mutated in the *cre1-1* mutant. Hereafter we refer to this gene as *CRE1*. *CRE1* is identical to *WOL*[15] (see below) and *AHK4* (ref. 16). A genomic fragment containing *CRE1* was introduced into *cre1-1* mutant calli. Wild-type calli that had been transformed with the control vector regenerated shoots when cultured in the presence of the

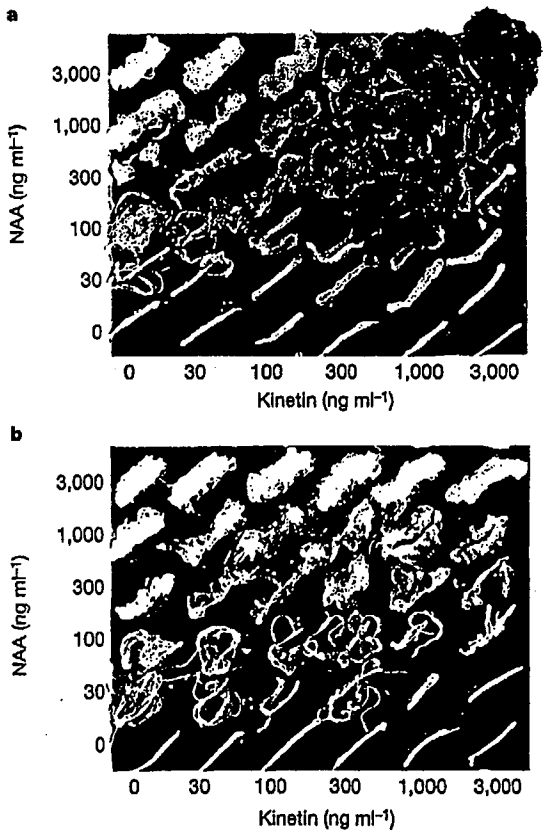

Figure 1 Callus growth of the cytokinin-resistant mutant *cre1-1* in different auxin and cytokinin concentrations. Hypocotyl segments were excised and cultured on media containing different levels of kinetin and NAA. After 21 days in culture, the induced calli were arranged and photographed. Wild-type explants (a) proliferated rapidly, turned green, and produced shoots in the presence of high concentrations of cytokinins. The *cre1-1* explants (b) did not.

cytokinin *trans*-zeatin, but *cre1-1* mutant calli transformed with the control vector did not (Fig. 3). However, mutant calli regenerated shoots in the presence of *trans*-zeatin if they had been transformed with pGPTV–KAN–CRE1 (Fig. 3d), indicating that *CRE1* complemented the *cre1-1* mutant.

We screened a library of *Arabidopsis* complementary DNA and isolated the corresponding cDNA clones, which were derived from two types of alternately spliced message named CRE1a and CRE1b. The predicted protein for CRE1a consists of 1,057 amino acids and that for CRE1b has 23 extra amino acids at its amino terminus. The CRE1a product was used for further study and for numbering of the amino-acid residues. The carboxy-terminal region of CRE1 carries a histidine kinase domain and a receiver domain. Between these domains, there is another region with weak similarity to receiver domains. According to the PSORT prediction (http://psort.nibb.ac.jp/form.html), CRE1 probably localizes to the plasma membrane. The N-terminal region probably consists of an extracellular domain flanked by two transmembrane segments, and the C-terminal region is intracellular. We detected CRE1 message in various tissues (data not shown), but the highest expression was in the root[15]. The *cre1-1* mutation converted Gly 467 in the histidine kinase domain to Asp 467 (see Supplementary Information). *Arabidopsis* has genes for two products, AAF99730 (AHK3 (ref. 16) and BAB09274 (AHK2 (ref. 16), that share high sequence similarity to CRE1, being 52% and 54% identical, respectively, over their entire proteins, and 61% and 60% identical, respectively, over their extracellular domains. CKI1 was less similar to CRE1 than these proteins (see Supplementary Information).

We also isolated an *Arabidopsis* line, *cre1-2*, with a T-DNA (see Methods) insertion in the *CRE1* gene. The integration occurred in the place of nine base pairs of *CRE1* between nucleotide positions +75 and +84 relative to the inferred translation initiation site (see Supplementary Information). The *cre1-2* line, which was homozygous for the T-DNA insertion, was also resistant specifically to cytokinins in the root elongation assay (Fig. 2) and in the callus growth and shoot formation assays (data not shown). The *cre1-2* mutant was complemented by introduction of the *CRE1* gene (see Supplementary Information). The presence of the mutation in the *CRE1* gene in either of the *cre1-1* or *cre1-2* mutants, and complementation of *cre1-1* and *cre1-2* by *CRE1*, are definitive evidence that mutations in *CRE1* cause the cytokinin-insensitive phenotype of the *cre1* mutants. The *cre1* mutants were allelic to the *wol* mutant (that is, the *cre1* and *wol* mutants bore mutations in the same gene), which is impaired in cell division and proper formation of vascular tissue of the root[15]. The xylem organization of *cre1-1* was also altered (data not shown).

To explore the function of the *CRE1* gene, we expressed *CRE1* (Fig. 4) in a yeast strain deficient in the *SLN1* gene, which encodes an osmosensing histidine kinase[10]. At normal osmolarity, SLN1 autophosphorylates the conserved histidine residue. The phosphoryl group is then transferred to the conserved aspartate residue in the receiver domain of the same protein, then to the phosphotransfer mediator YPD1, and finally to the SSK1 response regulator. This in turn inhibits the ability of SSK1 to activate the downstream mitogen-activated protein (MAP) kinase pathway[17]. The *sln1*Δ mutant is lethal because the downstream SSK1 is always dephosphorylated, which overactivates the downstream MAPK pathway[10,17]. The *sln1*Δ mutant carrying p415CYC–CRE1, which would express the *CRE1* gene, was still lethal without cytokinins. However, surprisingly, it grew at a normal rate if *trans*-zeatin, a native cytokinin, was included in the medium. It is noteworthy that the active cytokinin *trans*-zeatin[18] was effective in this yeast system, but the much less active cytokinin *cis*-zeatin[18] was ineffective (Fig. 4a). Other active cytokinins—isopentenyladenine, benzyl adenine and thidiazuron—were also effective. The plant hormones IAA, gibberellin A_3 and abscisic acid had no effect. Expression of CRE1b, another form of alternatively spliced product, in the *sln1*Δ mutant gave the same results (data not shown).

p415CYC–CRE1 did not suppress the lethality of the *ypd1*Δ mutant on plates either with or without cytokinins, indicating that

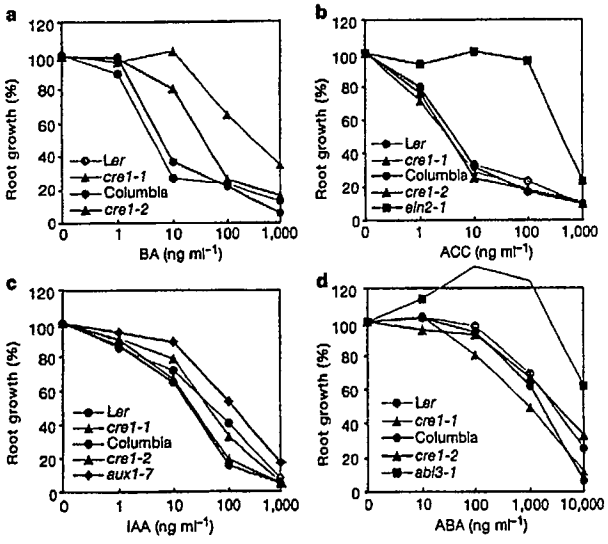

Figure 2 Root growth of the *cre1-1*, *cre1-2* and hormone-related mutants in the presence of various plant hormones. Seeds were sown on GM plates[22] containing BA (**a**), ACC (**b**) or IAA (**c**). After chilling for three days, plates were incubated at 23 °C for eight days and the root lengths were measured. To determine ABA response, two-day-old seedlings germinated on GM were moved onto plates containing different concentrations of ABA and cultured for six days (**d**). Root growth was expressed relative to the mean root elongation of the same genotype on the medium without plant hormones. Each value represents the mean of at least 11 plants. *cre1-1* and *abi3-1* have L*er* genetic background (red symbols); *cre1-2*, *ein2-1* and *aux1-7* have Columbia genetic background (black symbols). The root length of each genotype in the absence of plant hormones shown in **a**–**d** is given in the Supplementary Information.

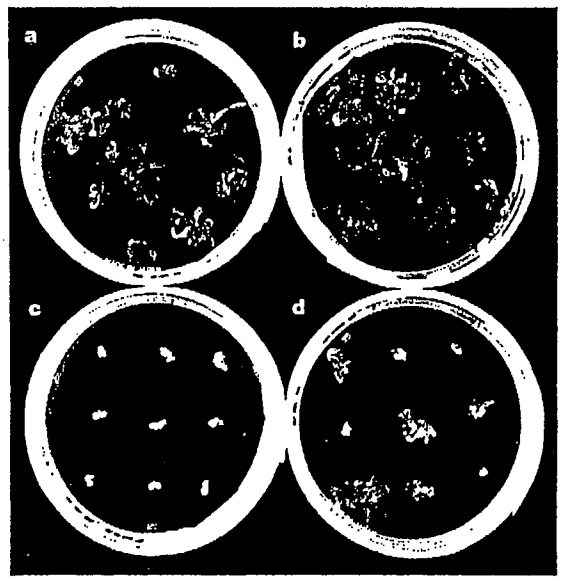

Figure 3 Complementation of *cre1-1* by *CRE1*. Wild-type calli (**a**, **b**) or *cre1-1* calli (**c**, **d**) were transformed with pGPTV–KAN (**a**, **c**) or pGPTV–KAN–CRE1 (**b**, **d**) and cultured for 19 days with 0.5 μg ml^{-1} *trans*-zeatin and 0.3 μg ml^{-1} indole butyric acid (an auxin). Shoots regenerated from different calli are independent transformants, and those on the same callus may or may not be independent.

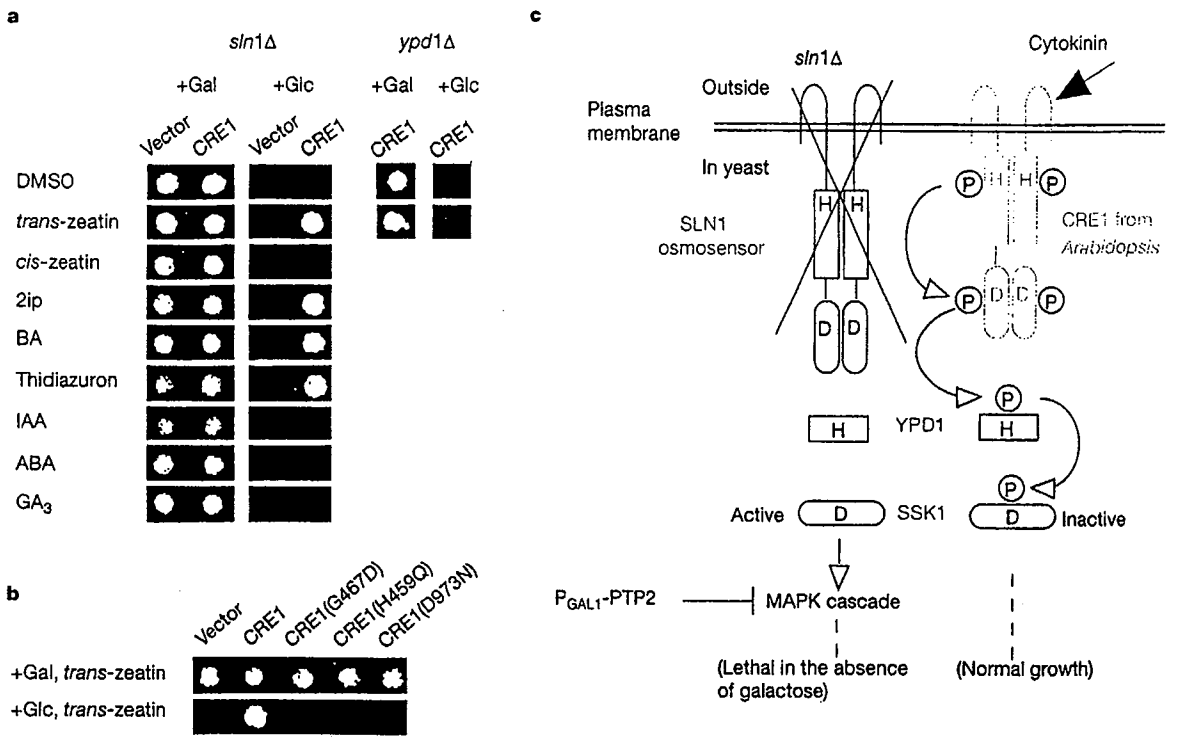

Figure 4 Cytokinin-dependent growth phenotype of yeast in which *SLN1* had been replaced with *CRE1*. **a**, *sln1*Δ and *ypd*Δ strains were transformed with the vector p415CYC (vector) or p415CYC–CRE1 (CRE1). Suspensions of transformants were spotted onto a plate containing a plant hormone as indicated, and galactose (+Gal) or glucose (+Glc). **b**, Effect of the mutation that was present in the *Arabidopsis cre1-1* mutant (G467D), or of the mutation that changed the conserved His 459 or Asp 973 phosphorylation sites (H459Q or D973N respectively). **c**, The presumed events in yeast. *CRE1* suppresses the lethality of *sln1*Δ but not *ypd1*Δ in the presence of cytokinins. The *sln1*Δ mutant is lethal because the dephosphorylated SSK1 constitutively activates the MAPK pathway. Cytokinins probably activate the histidine kinase activity of the CRE1 protein to initiate the phosphorelay, whereby the phosphoryl group is transferred from the activated CRE1 to YPD1, then to SSK1, suppressing the lethality of *sln1*Δ.

signal transduction from CRE1 is mediated by YPD1 in yeast (Fig. 4c). We next introduced the mutation that was present in the *Arabidopsis cre1-1* mutant into p415CYC–CRE1. The resulting plasmid, p415CYC–CRE1(G467D), could not suppress the lethality of *sln1*Δ either with or without *trans*-zeatin, indicating that the *CRE1* gene of the *Arabidopsis cre1-1* mutant was nonfunctional (Fig. 4b). Mutations at either the conserved His 459 or Asp 973 of the phosphorylation site in the histidine kinase or the receiver domains, respectively, also destroyed the ability of *CRE1* to suppress the lethality of the *sln1*Δ mutant (Fig. 4b). Therefore, cytokinins probably activate the histidine kinase activity of CRE1, and the signal is probably transmitted through YPD1 to SSK1. *Arabidopsis* also has phosphotransfer mediators[20,21], which resemble YPD1, and response regulators[4,5,7]. Therefore, in plants, cytokinins probably activate CRE1 and possibly its homologues, which in turn initiate the phosphorelay signalling that governs cytokinin responses.

In *Arabidopsis*, ethylene receptors[8,9] and possibly osmosensors[19] are histidine kinases. CKI1 histidine kinase has been implicated in the detection or signal transduction (or both) of cytokinins[3], but its function has yet to be clarified. We have provided evidence that the CRE1 histidine kinase is a cytokinin receptor: mutations in the *CRE1* gene caused a cytokinin-insensitive phenotype in *Arabidopsis*, and expression of *CRE1* conferred a cytokinin-responsive phenotype on yeast. The *cre1* and *wol* mutants were impaired in the cell division and differentiation that is essential for proper formation of the root vascular tissue[15]. This observation, coupled with our data, underlines the importance of cytokinin signalling in this process. The CRE1 homologues AAF99730 and BAB09274 may also function as cytokinin receptors, which may explain why defects in *CRE1* did not cause more diverse phenotypes related to cytokinin functions. □

Methods

Screening for mutants impaired in cytokinin responses

Seeds of *A. thaliana* ver. *Ler* were mutagenized with ethyl methanesulphonate, and seeds obtained after self-pollination (M2 seeds) were used[22]. Hypocotyl segments of M2 seedlings were aseptically excised and cultured on GM medium[23] supplemented with 100 ng ml^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D), 100 ng ml^{-1} of kinetin, and vitamins (100, 10, 1, 1 and 1 μg ml^{-1}, respectively, of inositol, thiamine, nicotinic acid, pyridoxine HCl and biotin). Kinetin of this concentration is sufficient to induce cytokinin responses in wild-type explants, including induction of calli with rapid growth and greening without forming root primordia. The top portion of the plant, corresponding to each hypocotyl segment, was grown on GM medium. Calli with a reduced green colour with many root primordia, which usually occur under low levels of kinetin, were chosen as mutant candidates, and their seeds were obtained by growing the corresponding top portions. From about 19,000 M2 seedlings, one line was confirmed for the heritability of the callus phenotypes. Root growth was measured on GM medium supplemented with a plant hormone as described in Fig. 2.

Transformation of calli

A genomic region encompassing the *CRE1* gene was amplified by polymerase chain reaction (PCR) from genomic DNA of *Ler* using primers 5′-AGCACAATGTGAGTTT-CACTGGCCTC-3′ and 5′-AGCTCAAGTCGTCGACTGAGCTATAG-3′. The amplified fragment was digested with *Sal*I and cloned into the pGPTV–KAN[25] vector between the *Sma*I and *Sal*I sites. The sequence of the resultant construct, pGPTV–KAN–CRE1, was confirmed and was transformed into *Arabidopsis* calli by the *Agrobacterium*-mediated method, as described[3,24], except that hormone concentrations of CIM medium[24] were changed to 0.5 μg ml^{-1} 2,4-D and 0.5 μg ml^{-1} kinetin. The transformed calli were cultured on GM medium supplemented with 50 μg ml^{-1} kanamycin sulphate, 100 μg ml^{-1} cefotaxime, 100 μg ml^{-1} vancomycin, 0.3 μg ml^{-1} indole butyric acid and 0.5 μg ml^{-1} *trans*-zeatin, and the same vitamins as were used in the medium for mutant screening. Other culture conditions were as described[3].

Screening for T-DNA insertion lines

We used the T-DNA insertion-line screening system organized at the Kazusa DNA Research Institute. The principles of the screening method were as described[25]. Gene-specific primers were 5′-ATATGCGATAGCGACTCTCGTACAA-3′ and 5′-AAC-CAAAATGCATATCAATCAGCAG-3′. T-DNA (pPCVICEn4HPT)[26] specific primers were 5′-ATAACGCTGCGGACATCTAC-3′ and 5′-ATCTAGGCTTTGATAGTCAC-3′. We used four combinations of primer sets, each consisting of a gene specific primer and a T-DNA-specific primer. The position of the T-DNA insert was determined by sequencing the PCR products carrying the T-DNA-genome junctions.

Yeast experiments

The entire coding region of the CRE1a cDNA was PCR-amplified and cloned into the yeast expression vector p415CYC[27] under the CYC1 promoter at the *Sma*I site, generating p415CYC–CRE1. We used the QuickChange site-directed mutagenesis kit (Stratagene) to generate p415CYC–CRE1(G467D), p415CYC–CRE1(H459Q) and p415CYC–CRE1(D973N). After sequence confirmation, plasmids were introduced into *sln1*Δ (strain TM182[10]) or *ypd1*Δ (strain SW100[17]). Suspensions of transformants were spotted (about 800 cells per spot) onto drop-out media with 10 μM plant hormones as indicated in Fig. 4, with either 2% glucose or 2% galactose.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Correspondence and requests for materials should be addressed to T.K. (e-mail: kakimoto@bio.sci.osaka-u.ac.jp). The accession numbers for CRE1a and CRE1b are AB049934 and AB049935, respectively.